Characterization of Trans-membrane Apparatuses in the Gram Negative Opportunistic Pathogens Nontypeable *Haemophilus influenzae* and *Acinetobacter baumannii*

by

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Table of Contents

List of Figures and Tables

Abstract

In today's world of ever evolving pathogens and rapidly acquired antibiotic resistance, the characterization of virulence factors has become increasingly important. Elucidating the mechanisms of action of these factors allows not only for a fundamental understanding of how microorganisms cause disease but also the identification of putative vaccine candidates. This work focuses primarily on trans-envelope molecular machinery in Gram-negative opportunistic pathogens, specifically, the type IV pili of nontypeable *Haemophilus influenzae* (NTHi) and the type VI secretion system of *Acinetobacter baumannii*. Each system mediates a variety of cellular processes and may be involved in the progression of disease in humans; therefore each is of great interest to the wellbeing of society. Here, we describe the characterization of components of NTHi type IV pili and *A. baumannii* type VI secretion in order to gain further insight into their biogenesis and function.

Dissertation Organization

This dissertation is organized into 4 chapters. Chapter 1 is an introduction containing background regarding nontypeable *Haemophilus influenzae,* type IV pili, *Acinetobacter baumannii* and type VI secretion. Chapter 2 describes the study of two nontypeable *H. influenzae* proteins, ComB and ComC, which are required for type IV pili production and/or function. Chapter 3 examines two genes, *tssB* and *tssD* and their products in the context of their role in bacterial competition via a type VI secretion system. General conclusions and ideas for future research are discussed in Chapter 4.

Chapter 1. General Introduction

Nontypeable *Haemophilus influenzae*

Background and History

Nontypeable *Haemophilus influenzae* (NTHi) is a Gram-negative bacterium that is part of the normal human microbiota, normally residing asymptomatically in the nasopharynx. Although NTHi is part of the normal microbiota, the microbe is able to cause disease under certain circumstances, and thus, is known as an opportunistic pathogen (36). NTHi is a significant causative agent of middle ear infections in children. There are currently no effective clinical treatments for middle ear infections caused by NTHi. The prevalence of middle ear infections in children and the lack of effective treatment options makes the development of new strategies necessary to reduce the prevalence of NTHi-induced infections (36).

Clinical Disease and Significance

NTHi can cause a variety of diseases such as pneumonia, sinusitis, exacerbations of chronic obstructive pulmonary disease and otitis media (OM). OM is a global health concern and a significant cause of morbidity in children, who are particularly vulnerable to OM due primarily to their immature immune systems and the anatomy of the juvenile Eustachian tube (36). In the United States, OM is the number one cause for pediatric visits to the emergency room and for prescriptions of antibiotics to children. Unfortunately, antibiotics have been found to be ineffective as a treatment for OM because of low drug bioavailability in the middle ear space and NTHi resides in the middle ear in a biofilm (2). The inherent antibiotic

resistance phenotype conferred by life within a biofilm, such as reduced growth rate and limited antibiotic penetration only adds the recalcitrance of NTHi-induced OM (2). Children that suffer from chronic OM that cannot be successfully treated with antibiotics often require tympanostomy tube insertion, in order to ventilate the middle ear cavity. This intervention is not a treatment, only an alleviation of symptoms associated with OM. Tympanostomy tube insertion requires the use of general anesthetic; consequently, OM is also the primary reason for the use of general anesthetic in children, which is itself a risk and can result in death (2).

Type IV Pili

Background and History

Type IV pili (Tfp) are dynamic surface appendages that are evolutionarily related to type II secretion systems and are produced by a variety of bacterial pathogens including *Neisseria* spp., *Salmonella* spp., and *Escherichia coli* (33). A collaboration between the laboratories of Dr. Robert S. Munson Jr. and Dr. Lauren O. Bakaletz demonstrated that NTHi express functional Tfp (3).

Type IV Pilus Mediated Phenotypes

Type IV pili mediate a variety of phenotypes; including twitching motility, adherence to surfaces, and competence. Twitching motility involves the repeated extension, attachment and retraction of the pilus fiber in an ATP dependent process. As stated before, pili can mediate attachment to surfaces, both abiotic and biotic. This can be a result of pili interacting directly with a host cell or abiotic surface or interactions between Tfp. The end product of this process is increased bacterial adherence and in the case of biotic surfaces, an increased chance of colonizing a potential host (41). Competence is the uptake of exogenous DNA, and

may contribute to virulence by allowing a microbe to take in foreign DNA as a nutrient source or, more critically, as a source of new genes (1).

Type IV Pilus Nomenclature

The nomenclature of Tfp systems is a quagmire. The most well characterized Tfp belong to members of the genera *Pseudomonas* and *Neisseria.* These systems have completely different naming conventions for each subunit of the Tfp complex, which requires intimate familiarity with each system or tables for cross-reference. For the purposes of this thesis, *Pseudomonas* nomenclature is used in describing what is known regarding Tfp machinery due it's similarly to a portion of the nomenclature for subunits that comprise NTHi Tfp.

Type IV Pilus Machinery

Type IV pili are dynamic macromolecular machines that span both the inner- and outer- membranes of Gram-negative bacteria. These machines are conserved across a number of bacterial genera and consist of a set of core proteins. Tfp can be categorized into two major groups, type IVa and type IVb, by the length and sequence of their major pilin subunits. Also in general, type IVa pili are generally produced by respiratory pathogens while type IVb pili are generally produced by enteric pathogens (10).

The major pilin subunit, PilA comprises the bulk of the Tfp pilus body. All type IV pilins are similar in structure with an extended N-terminal alpha-helical tail that connects the globular beta-sheet region as the head. The pilus filament is comprised of many PilA subunits that pack together in a helical arrangement through interactions with the N-terminal alpha-helical region (10).

The pilus extends through the outer membrane through a gated channel called the secretin. The secretin is a homo-multimeric complex comprised of, in most Tfp systems, 12-14 individual PilQ subunits. The stability of the secretin is affected a pilotin generally found in the inner-leaflet of the outer-membrane and by components of the Tfp inner-membrane machinery (1).

The Tfp inner-membrane complex functions to rapidly polymerize and depolymerize Tfp and is much more variable in structure than the outer-membrane complex. Type IVa pilus inner-membrane complexes, such as the case for *Pseudomonas*, usually contain a cytoplasmic protein associated with two bitopic inner membrane proteins, and an inner-membrane lipoprotein. Type IVb pili inner-membrane complexes generally consist of a polytopic platform protein, a variable number of bitopic inner-membrane proteins, and at least one ATPase.

The bitopic inner-membrane proteins of the *Pseudomonas aeruginosa* Tfp inner-membrane complex are PilN and PilO. These proteins have no known function, but are predicted to be involved in stabilization of the Tfp inner-membrane complex. Bitopic inner-membrane proteins often interact with each other and platform proteins. PilN and PilO were observed to form homo- and hetero-dimers. The platform protein, PilC, is hypothesized to function in the formation of the inner-membrane complex. PilP is the secretin dynamic-associated protein which interacts with the secretin and the two bitopic inner-membrane proteins. PilP is also believed to participate in gating of the secretin. ATPases are hexameric proteins that are required for functional Tfp and mediate the extension and retraction pili. Energy for extension and retraction is generated by the

hydrolysis of ATP molecules. There are three ATPases in Pseudomonas, PilB, PilT, and PilU (1).

NTHi Type IV Pili

The NTHi, strain 86-028NP genome (12) encodes two operons, *pilABCD* and *comABCDEF,* that are believed to encode the core set of proteins responsible for the biogenesis of a Type IVa pilus. The body of the pilus is comprised of the major pilin subunit encoded by *pilA* (1). *pilB* encodes the putative extension ATPase, which transfers energy in the form of ATP to extend the pilus from the periplasm through the outer-membrane into the extracellular environment. *pilC* encodes a putative Tfp inner-membrane platform protein. *pilD* encodes the major pilin peptidase, which cleaves the leader peptide from PilA and is necessary for Tfp production (unpublished, Munson).

The *com* operon of NTHi, strain 86-028NP has a similar arrangement to the *pilMNOPQ* operon which plays a role in type IV pilus biogenesis in *P. aeruginosa* (1). *comA* encodes a putative cytoplasmic protein of unknown function. The *comB* gene and the *comC* gene also encode proteins with no known functions. However, ComB and ComC share protein sequence similarity to the *P. aeruginosa* Tfp biogenesis proteins PilN and PilO, respectively. *comD* encodes a putative periplasmic protein that is thought to affect secretin multimerization. The secretin, encoded by *comE*, is an outer-membrane complex of multiple subunits of ComE that facilitates passage of the Tfp through the outer-membrane. Finally, *comF* encodes a protein of unknown function. Mutation in any one of these genes results in a loss of transformation, a phenotype linked to Tfp (7).

With regards to NTHi biology, Tfp have been shown to play a role in competence, twitching motility, biofilm formation, adherence to airway epithelial cells and colonization of a mammalian host (3, 7, 26, 27). NTHi PilA has proven to be an ideal target for the design of a vaccine to combat NTHi-induced OM. In a chinchilla model of NTHi-induced experimental OM, animals that were administered a PilA-based vaccine were protected from NTHi challenge. In addition, animals in which OM was induced prior to vaccine delivery exhibited less severe disease indicators and the infection was cleared sooner than in animals that received adjuvant alone. These results indicate both a therapeutic and preventative nature to a PilA-derived vaccine. This vaccine candidate has been formulated to be administered via a non-invasive transcutaneous regimen that demonstrates efficacy as both a therapeutic and preventative to target NTHi-induced OM (38-40).

Acinetobacter baumannii

Background and History

Acinetobacter baumannii is a Gram-negative opportunistic pathogen that has garnered much attention as the cause of nosocomial infections. *Acinetobacter* strains are known to be ubiquitous in the environment; however, the natural reservoir of *A. baumannii* is unknown. Recently, evidence has been presented that suggests this organism is able to persist both within human hosts and in health care facilities. Of greatest concern to the medical community, and what has most contributed to the emergence of this organism into the public spotlight, is *A. baumannii*'s rapid acquisition of multidrug resistance. Strains of *A. baumannii* that are resistant to every antibiotic currently in clinical use have been reported, making the discovery of novel treatment and prevention strategies imperative (44).

Clinical Disease and Significance

The natural ability of *A. baumannii* to survive harsh conditions such as prolonged periods of desiccation on inanimate surfaces, which are common in hospital elements such as furniture, medical equipment, as well as plastic and glass surfaces, contribute to the emergence of this organism as an important causative agent of nosocomial infections (24, 54). *A. baumannii* generally infects those with weak immune systems making children, the elderly, the wounded, and the immune-compromised particularly vulnerable. Most of the severe human infections caused by *A. baumannii* are thus acquired in health care facilities. *A. baumannii* is responsible for up to 10% of intensive care unit infections (15, 43, 51), up to 7% of pneumonias, and also causes infections of intravascular devices, ulcers, surgical sites and the urinary tract in patients (5, 25, 54). *A. baumannii* infections are associated with disturbingly high mortality rates, which range from 20% to more than 50% (18). Compounding the threat of *A. baumannii* is its ability to acquire and exhibit antibiotic resistance, which is thought to significantly contribute to the virulence of this pathogen (11, 13). The existence of pan-resistant strains of *A. baumannii* has been reported, which indicates we are approaching the end of the antibiotic era for this bacterium (19).

Type VI Secretion System

Background and History

Type VI secretion systems (T6SS) are trans-membrane molecular apparatuses that were first discovered in 2003 in *Rhizobium leguminosarum* (46). These systems are involved in the exportation of effector proteins and have been likened to needles or syringes, capable of injecting effectors directly into foreign cells. The ability of T6SS to secrete molecules in this

fashion is thought to derive from their structural relation to phage injection machinery. These similarities have caused T6SS to be depicted as inverted bacteriophage-like machines. Genes that encode T6SS are present in approximately 25% of all sequenced Gram-negative bacteria and are often associated with virulence and host infection by pathogens (49).

Type VI Secretion Related Phenotypes

Bacterial and eukaryotic cell targeting are the most fully characterized processes carried out by T6SS. Bacterial cell targeting allows T6SS producing organisms to compete with other bacteria, often by killing or inhibiting the growth of the foreign species (23, 32, 35, 45, 47). This allows organisms to be more successful at adapting to and surviving in their environments, which implies that T6SS could be relevant to the outcome of polymicrobial human diseases. The ability of T6SS to target eukaryotic cells may also affect a bacterial species capacity to be a pathogen. Numerous studies have shown that T6SS mutants are less virulent than wild-type strains in animal models of disease. The molecular basis for this phenotype is not well characterized (46); however, a series of experiments on the *Vibrio cholerae* virulence associated secretion system demonstrated that T6SS could affect macrophage cell rounding and intestinal inflammation in mice (46, 49).

Type VI Secretion Machinery

Type VI secretion systems traverse both the inner- and outer-membranes of Gram-negative bacteria. The genes that encode the components of T6SS are generally grouped in tightly clustered operons. An organism may carry more than one T6SS gene cluster. These clusters are usually divergent and likely the result of horizontal gene transfer. Thirteen conserved subunits compose the core T6SS apparatus. Aside from the 13 core

subunits, genes found within T6SS gene clusters may encode additional accessory proteins (6, 9).

The genes that encode the core components of T6SS can be organized into three categories, genes that encode inner-membrane proteins, bacteriophage related proteins, and proteins with no predicted function. The best understood T6SS belong to *Edwardsiella tarda* and *Vibrio cholerae*. The first group includes genes that code for TssL, TssM and TssJ. The second group includes the genes that code for Hcp, VgrG, TssB, TssC, and TssE, and the third group includes TssA, TssF, TssG, TssK (49).

TssL and TssM are localized to the inner-membrane and are homologous to IcmH and IcmF, which are components of the *Legionella pneumophilia* T6SS. Interestingly, IcmH and IcmF lack orthologs in other type IV secretion translocons (52). IcmH and IcmF interact with each other, therefore TssL and TssM are thought to interact with each other as well. The bulk of TssL is localized in the cytoplasm whereas TssM is localized in the periplasm. The cytoplasmic domain of TssM contains an ATP binding and hydrolysis motif. The function of this motif depends on the bacteria in which the system is present. TssJ a lipoprotein that is localized to the outer-membrane and interacts with TssM (49).

The two most-extensively characterized components of any T6SS are TssD (Hcp) and TssI (VgrG), which belong to the bacteriophage-related group of proteins. These proteins share secondary structure with phage injection constituents. TssD structurally resembles gpV, the major tail protein of phage λ. TssI assumes a structure similar to the gp27/gp5 complex, the tail spike of bacteriophage T4 used for membrane penetration. TssC assembles into a tubular structure that is similar to the T4 contractile sheath. This structure is large enough to enclose a tube composed of TssD subunits, and is therefore predicted to propel TssD out of

the cell upon contraction. TssE shares primary sequence homology to the T4 bacteriophage baseplate protein gp25 (49).

Acinetobacter baumannii Type VI Secretion System

In *A. baumannii* strain M2 a putative T6SS gene cluster, *asaA-tssBCDEFG-asaB-tssM-tagFN-asaC-tssHAKL-asaDE*, which extends over a 22 kb region, was identified. A nomenclature for the *A. baumannii* T6SS has been proposed (21) but differs significantly from the agreed upon standardized nomenclature for T6SS (48). The standard T6SS nomenclature will be followed in this dissertation. The genome of *A. baumannii* strain M2 was sequenced (data not shown) and the resultant contig set was screened for genes and/or predicted proteins that showed sequence identity to genes or proteins associated with other T6SS. This targeted search resulted in the identification of a gene cluster of 18 open reading frames and 3 additional genes that are predicted to encode 12 of the 13 core T6SS proteins (Tss), 2 proteins associated with T6SS in other bacteria (Tag) and 5 proteins that appear to be encoded only in *Acinetobacter* spp. (Asa). Three of these genes are predicted to encode orthologs of *tssI*. The predicted functions of many of the genes within the strain M2 T6SS gene cluster are unknown but are conserved amongst T6SS (21). However, there are functional predictions for the products of several genes within this gene cluster. In other bacteria, a homolog of the T4 phage baseplate must assemble in the inner- and outer-membrane prior to T6SS assembly (49). The product of the *tssD* gene shares sequence identity with a homolog of a protein found in the T4 bacteriophage baseplate. The *tssB* gene encodes a protein that share sequence identity with a subunit of the outer contractile sheath of T6SS characterized in other bacteria. The TssB homologue is required

for the physical extension of the inner core through the inner and outer membranes and subsequent secretion of effectors. The *tssD* gene encodes a protein, which has homology to gpV, the major tail protein of phage λ.

Summary with Goals and Hypothesis

The overall goal of this study was to further characterize the trans-membrane molecular machinery of NTHi and *A. baumannii*, specifically the Tfp of NTHi and the T6SS of *A. baumannii*. We hypothesized that the NTHi Tfp proteins ComB and ComC are oriented and interact similarly to PilN and PilO of *Pseudomonas*. We also hypothesized that *A. baumannii* produces a functional T6SS that mediates contact dependent bacterial competition.

Chapter 2. The NTHi Type IV Pilus-Associated Proteins, ComB and ComC are Bitopic Inner-Membrane Proteins that Interact

Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is one of the three primary opportunistic bacterial pathogens that are responsible for middle ear infections, or otitis media, in children. We have demonstrated that NTHi produce surface bound appendages called type IV pili and that NTHi Tfp play a role in competence, adherence to epithelial cells, biofilm formation, and colonization of the mammalian nasopharynx (7, 27). The core set of proteins that are believed to compose the Tfp apparatus in NTHi, strain 86-028NP are encoded by two operons, *pilABCD* and *comABCDEF*. These genes were identified by sequence similarity, presence of conserved domains (3) and analysis of the competence regulon (42, 53). Mutational analysis was also used to identify these genes (8). These analyses revealed that NTHi Tfp belong to the type IVa group of Tfp, based on the sequence and length of *pilA*. Although several of the genes responsible for Tfp biogenesis have been identified, little is known about the proteins encoded by these genes. We proposed to further investigate components of NTHi Tfp. Herein, we describe the experiments designed to elucidate the topology and interactions of the NTHi Tfp proteins ComB and ComC.

Materials and methods

Strains and culture conditions

NTHi, strain 86-028NP was grown either at 37˚C, 5% CO_2 on chocolate agar or at 37˚C in brain heart infusion broth (BHI) (Difco, Detroit, MI) to which 2 µg heme

(Sigma, St. Louis, MO) and 2 µg of ß-NAD (Sigma) were added per ml of medium (sBHI). *Escherichia coli* were grown on Luria agar (L-agar) or in Luria broth (L-broth) (Difco). In the case of alkaline phosphatase assays, *E. coli* was grown in RM medium, a minimal media which lacks a carbon source. RM media was supplemented with glucose or glycerol (17). Media were supplemented with spectinomycin (Sigma) at a concentration of 200 µg/ml, ampicillin (Sigma) at a concentration of 50 µg/ml and/or kanamycin (Sigma) at a concentration of 20 µg/ml as needed. IPTG (Sigma), a structural mimic of allolactose, was added at a final concentration of 0.5 nM to *E. coli* cultures to induce the T7 expression system when needed. Arabinose (Sigma) was added to *E. coli* cultures a final concentration of 0.2% to induce gene expression when required.

Bacterial Strain and Plasmid Construction

All strains and plasmids used in this study are listed in Table 1.1. In order to determine the orientation of ComC in the inner-membrane, translational fusions to the genes encoding green fluorescent protein (GFP) or alkaline phosphatase (PhoA) were made at specific amino acid residues such that each protein contained a fusion in the predicted cytosolic portion, periplasmic portion or at the C-terminal end of ComC. A set of 6 plasmids was constructed for expression of translation fusions to ComC, three constructs with translational fusions to GFP and three constructs with translational fusions to PhoA. Forward and reverse primers, both containing NheI restriction sites, were used to amplify region of *comC* from the beginning of *comC* to codons encoding specific amino acid residues on the 3' end. For ComC fusions, amplified regions contained the ATG start site and sequence encoding up to the 48 and 173 amino acid residues. The PCR products encoding each fragment were digested

with NheI and ligated with pBAD-GFP cut with NheI. Translational fusions between ComC with PhoA were constructed in the same manner as the *gfp* fusions but KpnI was used to restrict PCR products and these products were ligated into pBAD-PhoA precut with KpnI. Fusions encoding the first 15 amino acids of ComC to GFP or PhoA were constructed by PCR amplification of pBAD-GFP or pBAD-PhoA primers that contained sequence encoding the first 15 aa of ComC. These products were self-ligated. All ligation products were transformed into *E. coli* LMG194 and transformants plated on RM media with selection. *E. coli* clones harboring plasmids with fusions in the correct orientation were verified by sequencing.

Alkaline Phosphatase Activity

E. coli clones that contained pBAD constructs encoding truncations of ComC fused to PhoA were cultured in RM media supplemented with 0.2% glucose and grown to mid-log phase. Cultures were grown in the presence of glucose to inhibit expression of fusion proteins under the control of the *araBAD* promoter via catabolite repression. The cultures were then washed with RM media supplemented with 0.2% glycerol. Glycerol is a carbon-source that will not inhibit expression of genes under the control of the *araBAD* promoter. Expression of the translational fusions was induced by the addition of arabinose to a final concentration of 0.2%. Cells were harvested by centrifugation and subjected to an alkaline phosphatase activity assay as described by Duffy *et al.* (8).

Protein Expression and Purification

E. coli BL21 (DE3) (Invitrogen) cells carrying plasmids for expression of the 6xHIS-tagged periplasmic domains of ComB and ComC were cultured in L-Broth at 37˚C

with shaking. Cells were grown to an A_{600} of 0.6, at which time protein expression was induced. Expression was induced by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. The expression of the periplasmic domains of ComB and ComC was carried out at 19˚C with shaking for 4.5 h to encourage proper protein folding. Upon completion of induction, cultures were washed twice in PBS. Dithiobis[succinimydlpropionate] (DSP, Thermo Scientific), a nonpolar membrane permeable crosslinking agent, was dissolved in dimethylsulfoxide (DMSO) and added to cells resuspended in PBS to a final concentration of 2 mM. Cross-linking was carried out for 30 min at room temperature, then Tris, pH 7.5 was added to a final concentration of 20 mM to quench cross-linking. Cells were washed twice in PBS, resuspended in column loading buffer (Novagen) and lysed via bead disruption using B lysing matrix tubes (MP Biomedical). Cell lysate was centrifuged at 100,000xg to separate the soluble and insoluble fractions. Immobilized metal affinity chromatography (IMAC) was used to purify 6xHIS-tagged proteins from soluble cell lysate. Ni-NTA His-Bind resin (Novagen) was used following manufactures protocol for purification under denaturing conditions.

Protein Analysis and Identification

Concentration of protein in samples was determined and standardized using the bicinchoninic acid assay (Pierce, Rockford, IL) (35). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein samples by size. Samples were mixed with an equal volume of 2x Laemmli sample buffer with added 2-mercaptoethanol (BioRad, Hercules, CA), boiled for 10 min, cooled to room temperature and then resolved on 4-20% Mini-Protean TGX Precast gels (Bio-Rad). Resolved proteins were transferred from

SDS-PAGE gels to nitrocellulose for western blotting. Gels were blocked for 1 h with 2% bovine serum albumin. Rabbit polyclonal antibodies directed against ComB, residues 26-42, and ComC, residues 19-40, (Genscript, Piscataway, NJ) at a dilution of 1/4000 were used as primary antibodies in western blot analysis. The secondary antibody, an alkaline phosphate conjugated goat α-rabbit antibody (Invitrogen), was added at a dilution of 1:2500. Colorimetric detection with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride (BCIP/NBT)(Sigma) was used to verify the presence of protein.

Results and Discussion

ComB and ComC are Bitopic Inner-membrane Proteins

Experimental evidence has shown that PilN and PilO of *Pseudomonas aeruginosa*, homologs of ComB and ComC respectively, are bitopic inner-membrane proteins that comprise part of the Tfp machinery. Furthermore, Phyre2 structure prediction provided evidence that ComB and ComC may have similar structures to the PilN and PilO proteins from *Pseudomonas*. Thus, we hypothesized that ComB and ComC are also bitopic inner-membrane proteins with a similar topographical orientation as PilN and PilO. TMHMM was used to predict the topology of ComC based on secondary structure prediction (29). Translational fusions between green fluorescent protein (*gfp*) or alkaline phosphatase (*phoA)* at distinct residues in each protein, based on TMHMM topology predictions, see **Figure 1**, were utilized to determine topology. Translational fusions with *gfp* will emit green fluorescence when excited and when the GFP domain is located in the cytoplasm but not when the GFP domain is in the periplasm due to improper folding of GFP in this

compartment. Conversely, translation fusions with *phoA* will exhibit alkaline phosphatase activity when the PhoA domain is localized to the periplasm but not when the *phoA* domain is localized to the cytoplasm due to the lack of disulfide bond formation necessary for proper PhoA activity. This experiment demonstrated that ComC contains a larger periplasmic domain, a transmembrane domain, and a smaller cytoplasmic domain, results shown in **Figure 2A** and **2B**. These results are consistent with the model presented in **Figure 1B**. The topology mapping experiments concerning ComB, performed by Anna Bailey, yielded similar results (Data not shown).

Periplasmic Domains of ComB and ComC Interact

To study interactions between ComB and ComC, the soluble periplasmic domains of ComB and ComC were expressed in *E. coli*. The periplasmic domain of ComC with a C-terminal His tag fusion was co-expressed with the periplasmic domain of ComB. After co-expression, proteins were cross-linked with DSP and cells were lysed via bead beating and then subjected to centrifugation. IMAC was performed to purify 6xHIS tagged proteins from soluble cell lysate fractions. $ComB_{\Delta 2\text{-}35}$ and $ComC_{\Delta 2\text{-}42}$-HIS, when co-expressed, co-eluted at an imidazole concentration of 150 mM as evidenced by Western blots using antibodies specific to ComB or ComC. Western blots are shown in **Figure 3**. The co-expression of $ComB_{\Delta 2\text{-}35}$-HIS and $ComC_{\Delta 2\text{-}42}$ did not yield interpretable results due to nonspecific binding of $ComC_{\Delta 2\text{-}42}$ to the nickel columns. Samples were also visualized by Coomassie staining. Stained gels exhibited bands corresponding to the approximate molecular mass of hetero-dimers of $ComB_{\Delta 2\text{-}35}$ and $ComC_{\Delta 2\text{-}42}$-HIS as well as hetero-dimers of $ComB_{\Delta 2\text{-}35}$-HIS and $ComC_{\Delta 2\text{-}42}$. Putative hetero-dimer bands were excised and submitted

for mass spectroscopy analysis. Peptides, which were identified by mass spectroscopy, are indicated in red in **Figure 4**. These peptides correspond to the periplasmic domains of ComB and ComC. These data indicate an interaction between the NTHi Tfp proteins ComB and ComC and further support the hypothesis that they have similar functions as PilN and PilO of *Pseudomonas.*

Concluding Remarks

The data generated from these experiments demonstrated that ComB and ComC are both bitopic inner-membrane proteins and can interact as heterodimers via their periplasmic domains. These data fill gaps in our understanding of NTHi Tfp structure and biogenesis.

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Chapter 3. The *A. baumannii* Type VI Secretion-Associated Genes, *tssB* and *tssD*, Play a Role in Inter-Bacterial Competition

Introduction

Acinetobacter baumannii is a Gram-negative, nosocomial pathogen that causes pneumonia, surgical site infections, bacteremia, and necrotizing fasciitis, among other diseases in humans. In addition to the list of diseases caused by *A. baumannii*, many strains are highly antibiotic resistant. These two facts coupled together make the study of virulence factors in *A. baumannii* absolutely crucial, as the information gained may someday go towards new treatment development.

A clinical isolate of *A. baumannii*, designated strain M2 (37), carries a gene cluster predicted to encode a type VI secretion system (unpublished, Carruthers). T6SS are bacterial secretory apparatuses that are analogous to bacteriophage injection machinery (49). Secretion systems such as T6SS allow bacteria to transport macromolecules and effector proteins into their environment or directly into other cells (4). As the products of these genes are poorly understood and no function has been ascribed to a T6SS produced by *A. baumannii*, we endeavored to investigate their function. This chapter describes the experiments designed to elucidate the role of *tssB* and *tssD* in *A. baumannii* strain M2 and determine the role of the *A. baumannii* T6SS.

Materials and methods

Strains and culture conditions

All strains and plasmids are listed in Table 2.1. *A. baumannii* strain M2, a clinical isolate from Cleveland Metro-Health Systems obtained from Phil Rather at Emory University, was used in this study (37). All bacterial strains were grown on L-agar or L-broth prior to experimentation. *A. baumannii* cultures, where appropriate, were supplemented with kanamycin20 µg/ml; chloramphenicol 12.5 µg/ml; spectinomycin 200 µg/ml or ampicillin 750 µg/ml. *Escherichia coli* cultures were supplemented with kanamycin 20 µg/ml or ampicillin 50 µg/ml, where appropriate.

Bacterial Strain Construction

A. baumannii strain M2 clones with unmarked mutations in *tssB* and *tssD* were constructed using a recombineering strategy as described by Tracy *et al* (50). The kanamycin resistance cassette from Tn*903* and *sacB*, flanked by FRT sites, were amplified from pRSM3542, a plasmid constructed by the Munson lab group. In the case of constructing the *tssB* mutant the resultant PCR product contained 47 bp upstream of *tssB*, the ATG start codon, an FRT site, the *sacB*-kan cassette, a second FRT site, the last 21 bp of *tssB* including the stop codon and 29 bp down-stream of *tssB.* A similar PCR product was made in the case of *tssD*. Plasmids that carried *tssB* and *tssD* and approximately 1 kb of flanking sequence were constructed by ligating PCR products generated from PCR with M2 genomic DNA to pGEM-T-Easy (Promega). These ligated products were transformed via electroporation into *E. coli* DH5a and kanamycin resistant colonies were selected. These plasmids were purified from *E. coli* DH5a, mixed with the amplicons containing *sacB*-kan flanked by 50 bp of

homology, and electroporated into *E. coli* DY380 cells that had been heat shocked at 42˚C for 15 min in order to carry out homologous recombination. Plasmids were purified from kanamycin resistant clones and verified by sequencing. Plasmid from single clones were saved as pGEM-Δ*tssB*::*sacB*-kan and pGEM-Δ*tssD*::*sacB*-kan. Amplicons containing *sacB*-kan and 1 kb of flanking DNA 5' and 3' of the cassette were generated from pGEM-Δ*tssB*::*sacB*-kan and pGEM-Δ*tssD*::*sacB*-kan. One microgram of these amplicons was mixed with 500 µl of M2 cultures grown for 2 h from a 1:10 dilution of an overnight culture for natural transformation. These mixtures were plated on L-agar and incubated at 37˚C for 4 h. Cells were then scraped from the plate and re-suspended in 500 µl of L-broth. Serial dilutions were performed and dilutions were plated on L-agar containing kanamycin to select for *Acinetobacter* mutants. Mutants were verified by PCR and sequencing. To obtain unmarked M2 mutations, *E. coli* DH5a harboring pFLP2, *E. coli* HB101 harboring pRK2013 and mutant M2 strains were used as the donor, helper and recipient strains respectively in a tri-parental conjugation. Unmarked mutants were selected for on L-agar containing chloramphenicol and 10% sucrose at room temperature. Sucrose resistant clones were analyzed by PCR to verify loss of the *sacB*::kan cassette. Correct clones were sequenced and saved as M2Δ*tssB* and M2Δ*tssD*.

TssD Secretion Assay

A. baumannii strain M2 was inoculated into 20 mL of L-broth at an A_{600} of 0.10 and grown at 37˚C with shaking until an A_{600} of 2.50 reading was reached. Cultures were centrifuged at 4,000 x *g*, 4˚C for 20 min to remove cells. The supernatant was centrifuged at 100,000 x *g*, 4˚C for 2 h to remove any insoluble material. Twenty milliliters of cleared

supernatants were concentrated by filtration through Ultracel 10K centrifuge filters (Amicon) according to the manufacturer's protocol. SDS-PAGE and Coomassie staining were utilized to analyze the concentrated culture supernatants. Bands that correlated to the approximate size of TssD were excised and sent to The Ohio State University Mass Spectrometry and Proteomics Facility for identification.

Bacterial Competition

A. baumannii strains were inoculated into 25 mL of L-broth at an A_{600} of 0.10 and grown at 37˚C with shaking. Once an A_{600} of 0.60 was reached cultures were diluted to an A_{600} of 0.40. For competition, *A. baumannii* strains were mixed at a 10:1 ratio to *E. coli* DH10B, 40 µL to 4 µL, and 20 µL of these mixtures were spotted onto L-agar. Competition was allowed to proceed for 4 h at 37˚C. After competition, spots were excised, submerged in 500 µL of PBS, vortexed for 3 s and serial diluted. Dilutions out to 10^{-6} were plated on L-agar containing streptomycin to select for *E. coli*.

T6SS Contact Dependence

The contact dependence assay was carried out in the same manner as the bacterial competition assay except 0.22 µm pore polycarbonate filters were placed between strains and/or media and dilutions out to 10^{-7} were plated.

Results and Discussion

Acinetobacter baumannii strain M2 Secreted TssD

Functional T6SS are known to produce and secrete homologs of gpV. To determine if *A. baumannii* produces a functional T6SS, the secretion of the gpV homolog in *A. baumannii*, TssD, was assayed for. *Acinetobacter* strains were grown to late log phase in

L-broth. The cells were then removed from the supernatant, which was subsequently concentrated. Concentrated supernatants were subjected to SDS-PAGE and stained using Coomassie Blue as seen in **Figure 5.** The data show that a secreted product in the range of 25-37 kDa was not present in the supernatant of either of the *Acinetobacter tss* mutant cultures. The protein band visible between the 25 and 37 kDa markers was submitted the Ohio State Mass Spectrometry and Proteomics Corps and identified as TssD. The presence of TssD in culture supernatants indicated that the *A. baumannii* strain M2 T6SS was functional and active during growth in culture.

Acinetobacter baumannii Utilized a T6SS for Inter-bacterial Competition

Bacterial T6SS are often associated with competition; they mediate the killing of other bacteria and can thus provide a growth advantage over would be competitors. Because the results of the secretion assay indicated that *A. baumannii* produced a functional T6SS the next logical step was to test whether that T6SS could facilitate the killing of other bacterial cells. In order to test this hypothesis, a bacterial competition assay between *A. baumannii* strain M2 and *E. coli* DH10B was conducted. *A. baumannii* strain M2 and *E. coli* DH10B were mixed at a 10:1 ratio, spotted onto L-agar, and after 4 hours of incubation spots were excised, homogenized in L-broth, diluted, and plated on agar selective for *E. coli*. The results shown in **Figure 6** demonstrated that strain M2 was able to out-compete *E. coli* cells as compared to the no *Acinetobacter* control. The Δ*tssB* and Δ*tssD* mutants showed a lack of phenotype, which indicated that killing was T6SS mediated.

Acinetobacter baumannii T6SS Mediated Competition was Contact Dependent

To establish whether the competition phenotype was contact dependent, a competition experiment with 0.22 µm pore filters was performed. When mixed together and spotted above and below a filter, as seen in **Figure 7.** *A. baumannii* strain M2 out-competed *E. coli* DH10B. It is also evident from this experiment that the location of *E. coli*, either above or below the filter, does not impact the amount of recoverable *E. coli*. No killing of *E. coli* was observed when a filter separated the two species. These data supported that *A. baumannii* competes with other bacterial strains in a contact dependent manner.

Concluding Remarks

The results of the experiments outlined in this chapter indicated that *A. baumannii* strain M2 produced a functional T6SS, as evidenced by the *tssD* secretion assay, that *A. baumannii* used this system to compete with *E. coli* DH10B, and that competition was contact dependent. In addition, our data is the first to ascribe a function to the T6SS produced by *A. baumannii,* namely *A. baumannii* uses its T6SS to compete with other bacterial species in a contact dependent manner.

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Chapter 4. General Conclusions and Future Directions

NTHi and *A. baumannii* cause a number of human diseases and are difficult to manage with conventional antibiotic therapies. Due to the severity of the diseases associated with these pathogens, new treatment options must be explored. Here, we examined components of NTHi Tfp and *A. baumannii T6SS*. The inner-membrane proteins, ComB and ComC, of NTHi were topology mapped and shown to interact. *A. baumannii* was shown to produce a functional T6SS capable of facilitating bacterial competition in a contact dependent manner. Future experiments that may further characterize these virulence factors include studying the structures and interaction sites of ComB and ComC via x-ray crystallography and assessing the role of *A. baumannii* T6SS in a mouse model. The data discussed in this study lay the foundation for future research that may someday be incorporated into the development of new treatment strategies for infections caused by the Gram-negative opportunistic pathogens NTHi and *A. baumannii*.

Appendix A. Tables

Table 1.1 Plasmids and Bacterial Strains

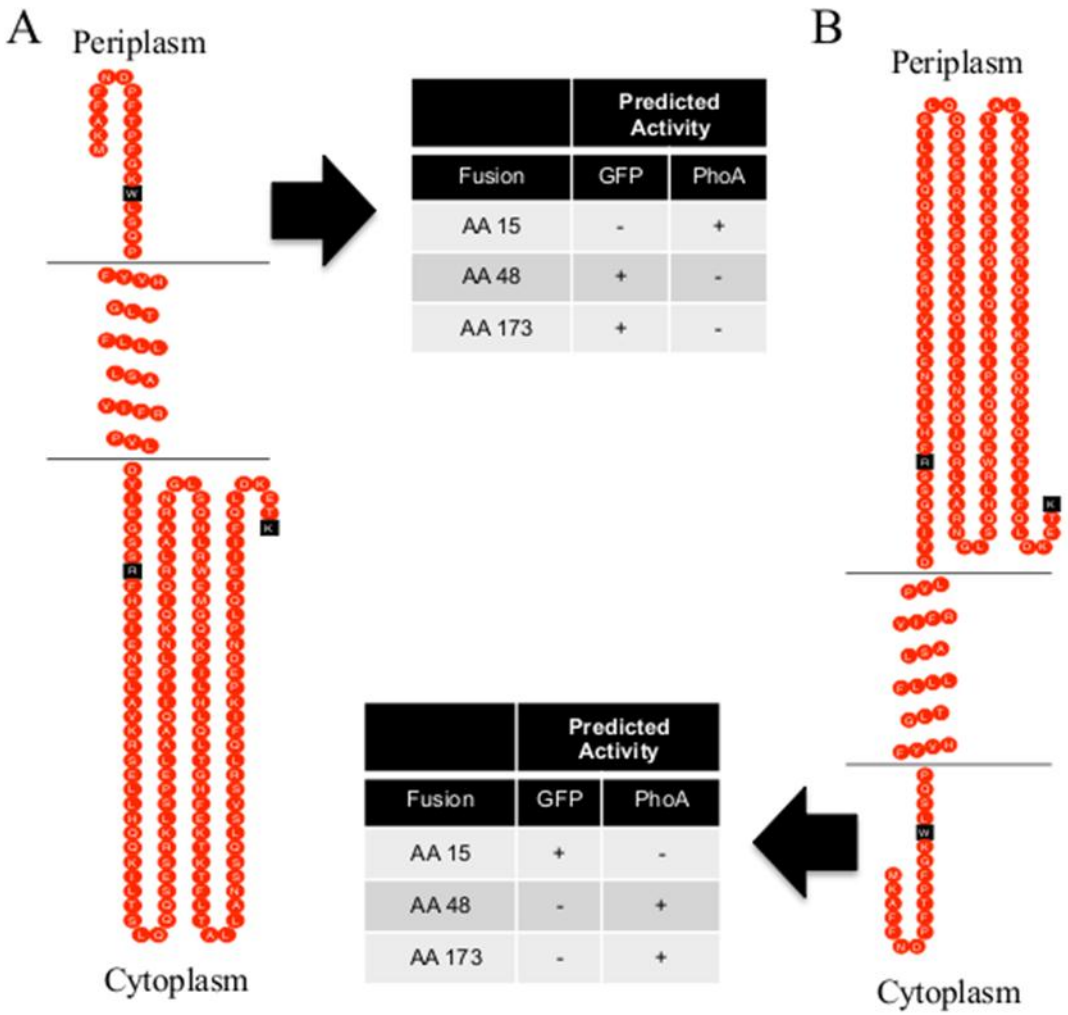
Plasmid or strain	Relevant characteristic(s)	Reference/ Source
Plasmids		
pGEM-TEasy	General purpose cloning vector	Promega
pSmartLCKan	Low copy cloning vector	Lucigen
pCOLA-Duet	*E. coli* expression vector	Novagen
pET-Duet	*E. coli* expression vector	Novagen
pBAD-PhoA	An arabinose inducible expression vector that allows for C-terminal fusion to PhoA	(34)
pBAD-GFP	An arabinose inducible expression vector that allows for C-terminal fusion to GFP	(31)
pComB-PhoA	pBAD with sequence encoding ComB with a C-terminal fusion to PhoA	This study
pComC$_{\Delta 15\text{-}178}$-PhoA	pBAD with sequence encoding ComC$_{\Delta 15\text{-}178}$ with a C-terminal fusion to PhoA	This study
pComC$_{\Delta 48\text{-}178}$-PhoA	pBAD with sequence encoding ComC$_{\Delta 48\text{-}178}$ with a C-terminal fusion to PhoA	This study
pComC-PhoA	pBAD with sequence encoding ComC with a C-terminal fusion to PhoA	This study
pComC$_{\Delta 15\text{-}178}$-GFP	pBAD with sequence encoding ComB$_{\Delta 51\text{-}168}$ with a C-terminal fusion to GFP	This study
pComC$_{\Delta 48\text{-}178}$-GFP	pBAD with sequence encoding ComB with a C-terminal fusion to GFP	This study
pComC-PhoA	pBAD with sequence encoding ComC with a C-terminal fusion to PhoA	This study
pComC$_{\Delta 15\text{-}178}$-GFP	pBAD with sequence encoding ComB$_{\Delta 51\text{-}168}$ with a C-terminal fusion to GFP	This study
pComC$_{\Delta 48\text{-}178}$-GFP	pBAD with sequence encoding ComB with a C-terminal fusion to GFP	This study
pComC-GFP	pBAD with sequence encoding ComC$_{\Delta 15\text{-}178}$ with a C-terminal fusion to GFP	This study
pComC$_{\Delta 2\text{-}42}$	pCOLA-Duet with sequence encoding ComC$_{\Delta 2\text{-}42}$	This study
pComC$_{\Delta 2\text{-}42\text{-}HIS}$	pCOLA-Duet with sequence encoding ComC$_{\Delta 2\text{-}42\text{-}HIS}$	This study
pET-ComB$_{\Delta 2\text{-}35}$	pET-Duet with sequence encoding ComB$_{\Delta 2\text{-}35}$	This study
pET-ComB$_{\Delta 2\text{-}35\text{-}HIS}$	pET-Duet with sequence encoding ComB$_{\Delta 2\text{-}35\text{-}HIS}$	This study
pComC$_{\Delta 2\text{-}42}$	pCOLA-Duet with sequence encoding ComC$_{\Delta 2\text{-}42}$	This study
pComC$_{\Delta 2\text{-}42\text{-}HIS}$	pCOLA-Duet with sequence encoding ComC$_{\Delta 2\text{-}42\text{-}HIS}$	This study
pET-ComB$_{\Delta 2\text{-}35}$	pET-Duet with sequence encoding ComB$_{\Delta 2\text{-}35}$	This study
pET-ComB$_{\Delta 2\text{-}35\text{-}HIS}$	pET-Duet with sequence encoding ComB$_{\Delta 2\text{-}35\text{-}HIS}$	This study
Strains		
Nontypeable *H. influenzae* strain 86-028NP	Clinical isolate from a child with OM	(28)
E. coli BL21 Star DE3	Strain for overexpression of *com* proteins in *E. coli*	Invitrogen
E. coli DH5∝	Cloning strain	Invitrogen
E. coli LMG194	*phoA*$^{-}$ strain used for alkaline phosphatase activity assays	(20)
E. coli TOP10	Cloning strain	Invitrogen

Table 2.1 Plasmids and Bacterial Strains

Plasmid or strain	Relevant characteristic(s)	Reference/Source
Plasmids		
pFLP2		(22)
pRSM574	pBR322 with Tn*903* kanamycin resistance cassette	This Study
pRSM2724	pRSM574 with *sacB* from pFLP2, cloned via EcoRI	This Study
pKD13		(12)
pGEM-T-Ez	General cloning plasmid	Promega
pGEM-*tssB*	pGEM, *tssB* region from strain M2	This study
pGEM-Δ*tssB*::*sacB*-kan	pGEM-*tssB*, deletion/insertion of *sacB*::kan cassette	This study
pGEM-*tssD*	pGEM, *tssD* region from strain M2	This study
pGEM-Δ*tssD*::*sacB*-kan	pGEM-*tssD*, deletion/insertion of *sacB*::kan cassette	This study
pSMART LC-Kan	General cloning plasmid	Lucigen
pRSM3542	pKD13, *sacB*::kan	This study
Strains		
Acinetobacter baumannii strain M2	Metro Health Systems Clinical Isolate	(37)
Δ*tssB*	*Acinetobacter baumannii* strain M2 mutant containing a unmarked deletion of *tssB*	This study
Δ*tssD*	*Acinetobacter baumannii* strain M2 mutant containing a unmarked deletion of *tssD*	This study
Δ*tssB*::-*sacB*-kan		
Δ*tssD*::-*sacB*-kan		This study
Escherichia coli DH10B	General cloning strain, competitor	Invitrogen
Pseudomonas aeruginosa PA01	Cm^S	(55)
E. coli DH5a	General cloning strain	Invitrogen
E. coli EC100D	General cloning strain, pir^+	Epicentre
E. coli DY380	Recombineering strain	(30)
E. coli HB101(pRK2013)		(14)

Appendix B. Figures

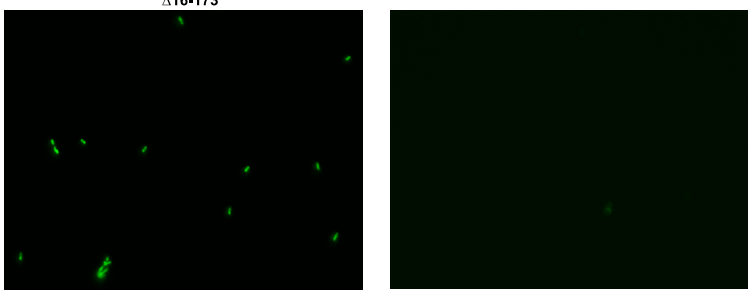
Figure 1. Predicted Topology Maps of ComC in the Inner-Membrane



A. Candidate topology map of ComC. This map show ComC with small periplasmic domain, a trans-membrane domain and a large periplasmic domain **B.** Candidate topology map of ComC. This map shows ComC with large periplasmic domain, a trans-membrane domain and a small periplasmic domain. The predicted activities of fusions between ComC and either Gfp or PhoA at specific amino acids are indicated in each panels corresponding table. Residues at which PhoA or GFP were fused to ComC are shown in black.

Figure 2. ComC is a Bitopic Inner-Membrane Protein

A.

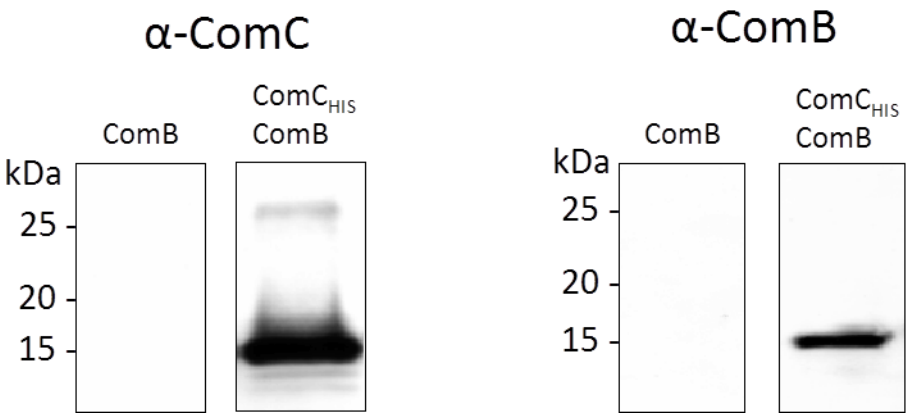


B.

ComC-PhoA Fusion at Residue:	PhoA Activity
15	-1.7
48	389.8
173	99.0

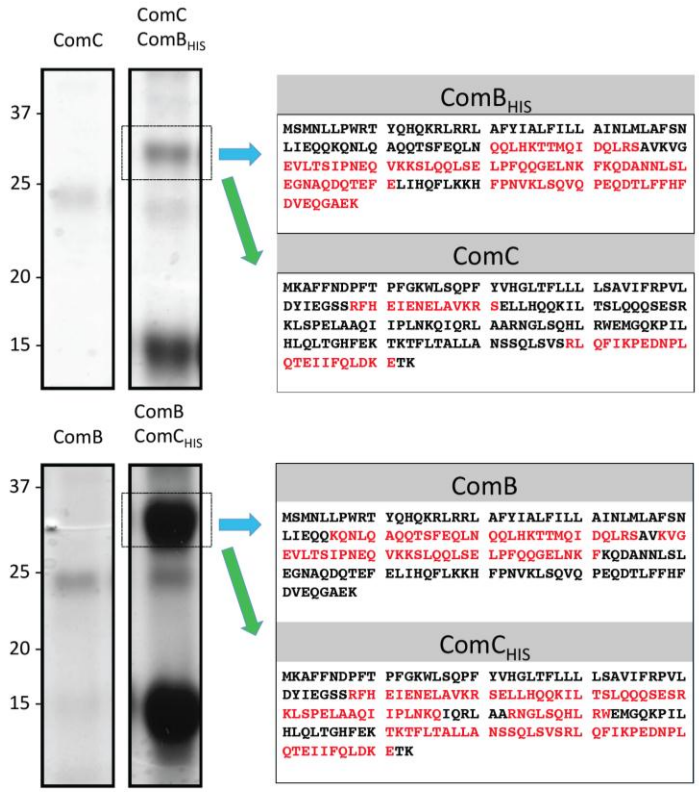
A. Cultures of *E. coli* expressing fusions of GFP to ComC at specific amino acids were tested to determine ComC localization and orientation in the inner-membrane. *Escherichia coli* that expressed the first 15 aa of ComC with GFP fused to the C-terminus (ComC$_{\Delta 16\text{-}173}$-GFP) were fluorescent, while *E. coli* that expressed full-length ComC with GFP fused to the C-terminus were not fluorescent. As GFP is active only in the cytoplasm these data indicated residues 1-15 of ComC are located in the cytoplasm while the C-terminal residue is not. **B.** Cultures of *E. coli* expressing fusions of PhoA to ComC at specific amino acids were tested to determine ComC localization and orientation in the inner-membrane. As PhoA is only active in the periplasm, these results indicated that ComC residues 48 and 173 are located in the periplasm, while residue 15 is not. These data suggest that ComC is a bitopic inner-membrane protein.

Figure 3. The Periplasmic Domains of ComB and ComC Interacted



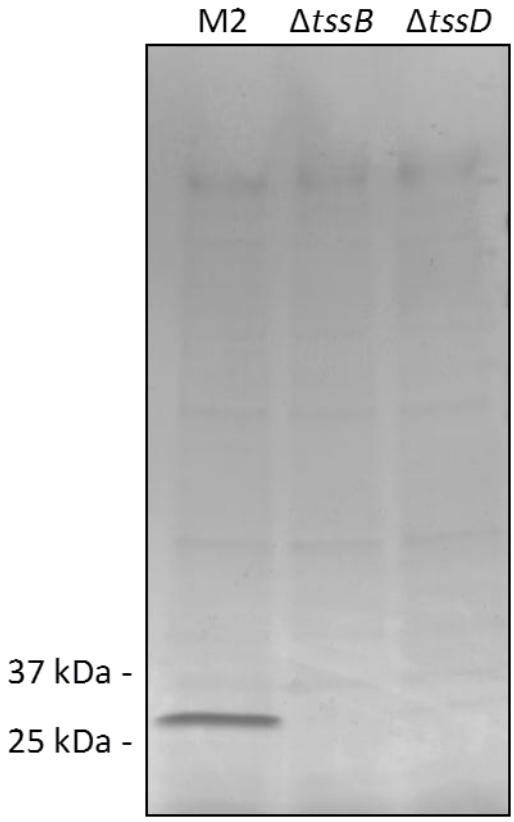
When the culture reached and OD_{600} of ~0.6, expression of the periplasmic portions of ComB and ComC was induced via the addition of IPTG and cultures were grown for an additional 4.5 h at 19˚C. Protein was cross-linked with DSP, cell were lysed via mechanical disruption and the cell lysate was centrifuged at 100,000 x *g* for 1 h. Soluble protein was purified by IMAC under native conditions and SDS-PAGE of purified fractions followed by Western blot analysis was performed. Western blot analysis indicated that ComC-HIS was purified by IMAC and that ComB was detected in pull-downs when purifying ComC-HIS. These data indicated an interaction between the periplasmic domains of ComB and ComC.

Figure 4. Analysis of Com Complexes by Mass Spectrometry



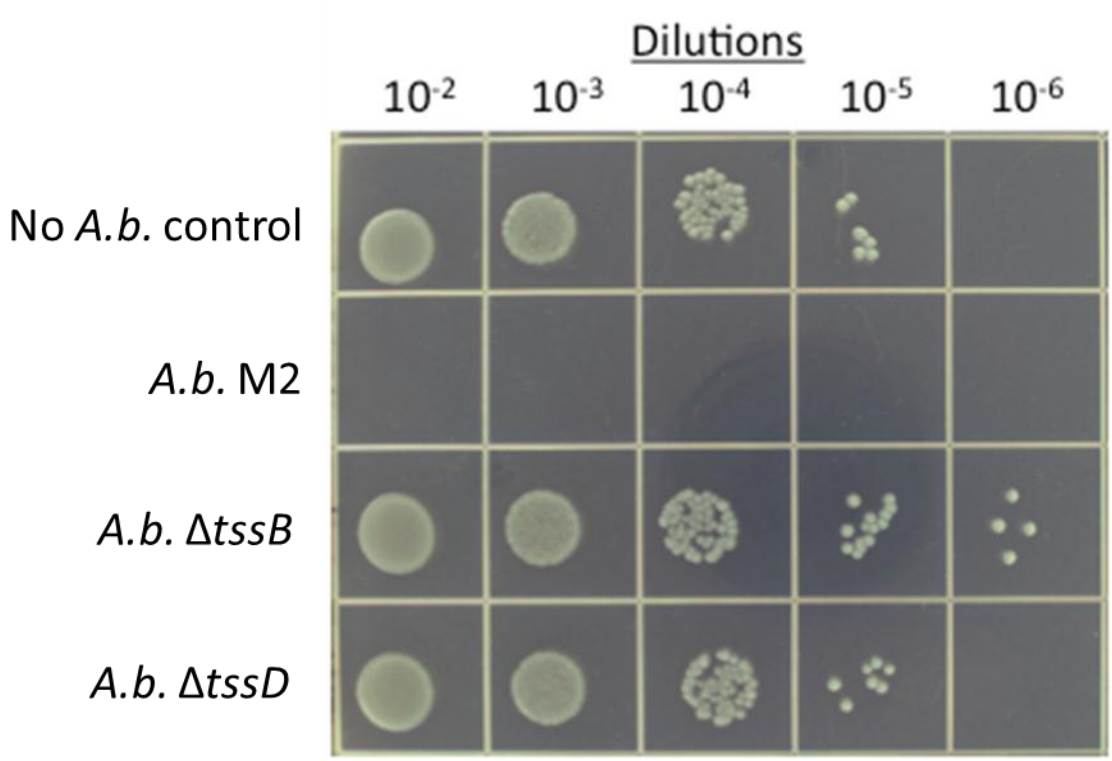
Escherichia coli BL21 (DE3) cells containing plasmids encoding the proteins described above were grown to early-log phase in LB and IPTG was added to induce expression of the periplasmic domains of ComB and ComC. Cultures were incubated overnight at 19°C. Protein was cross-linked with DSP and the cells were resuspended in denaturing buffer (SDS). Cells were lysed via mechanical disruption, lysate spun at 100,000 x *g* to separate soluble and insoluble proteins. Soluble proteins were subjected to IMAC under denaturing (SDS) conditions. Samples were resolved by SDS-PAGE was performed and gels were stained with Coomassie blue. The bands between 25 and 37 kDa that corresponded to potential heterodimers, as indicated by dashed boxes, were excised and submitted to The Ohio State Mass Spectrometry Facility for analysis. Amino acid sequences are displayed with identified peptides highlighted in red.

Figure 5. TssD is Secreted in Strain M2 and Not in the *tssB* and *tssD* Mutants



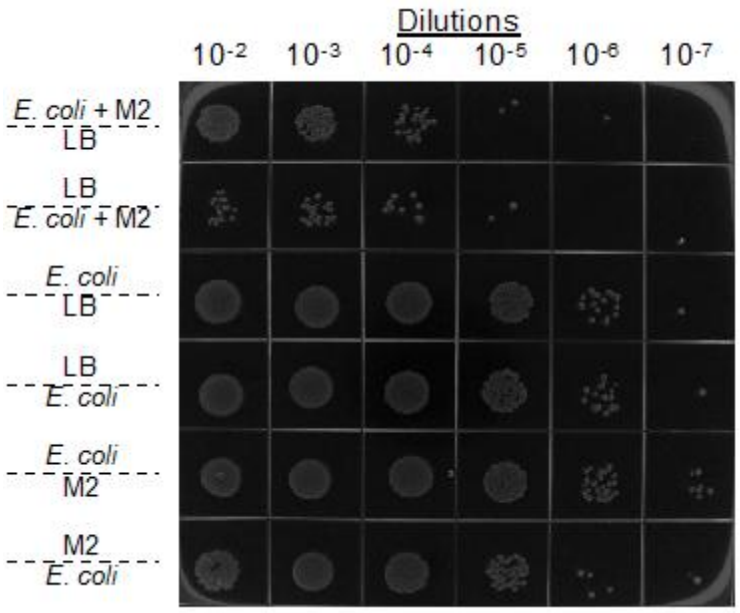
Culture supernatants of *A. baumannii* strains grown till early stationary phase at 37˚C, 180 rpm were cleared by centrifugation and ultra-centrifugation. Cleared supernatants were then concentrated 20-fold using a protein concentrator with a 10 kDa cut-off. A band with the approximate molecular mass of TssD was observed in the sample derived from strain M2 and was absent in the samples from the *tssB* and *tssD* mutants. Mass spectrometry confirmed that the most abundant protein the observed band from the M2 sample was TssD.

Figure 6. *Escherichia coli* was Out-Competed by *A. baumannii* in a T6SS-Dependent Manner



Escherichia coli DH10B and *A. baumannii* strains indicated were competed against one another at a ratio of 1:10 respectively for 4 h. Competition mixtures were plated on L-agar supplemented with streptomycin at 25 ug/mL to select for the surviving *E. coli* population.

Figure 7. Competition of *A. baumannii* Against *E. coli* was Contact Dependent



Escherichia coli DH10B cells plated after a 4 h competition with *A. baumannii* strain M2. The dashed line between strains and/or media signifies the presence of a 0.22 µm pore filter between the two bacterial populations or between bacteria and media.

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